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Inositol Lipid Binding and Membrane Localization of Isolated Pleckstrin Homology (PH) Domains

STUDIES ON THE PH DOMAINS OF PHOSPHOLIPASE C δ_1 AND p130*

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The relationship between the ability of isolated pleckstrin homology (PH) domains to bind inositol lipids or soluble inositol phosphates *in vitro* and to localize to cellular membranes in live cells was examined by comparing the PH domains of phospholipase Cδ_1 (PLCδ_1) and the recently cloned PLC-like protein p130 fused to the green fluorescent protein (GFP). The prominent membrane localization of PLCδ_1PH-GFP was paralleled with high affinity binding to inositol 1,4,5-trisphosphate ($InsP_3$) as well as to phosphatidylinositol 4,5-bisphosphate-containing lipid vesicles or nitrocellulose membrane strips. In contrast, no membrane localization was observed with p130PH-GFP despite its $InsP_3$ and phosphatidylinositol 4,5-bisphosphate-binding properties being comparable with those of PLCδ_1PH-GFP. The N-terminal ligand binding domain of the type I $InsP_3$ receptor also failed to localize to the plasma membrane despite its 5-fold higher affinity to $InsP_3$ than the PH domains. By using a chimeric approach and cassette mutagenesis, the C-terminal α-helix and the short loop between the β6–β7 sheets of the PLCδ_1PH domain, in addition to its $InsP_3$-binding region, were identified as critical components for membrane localization in intact cells. These data indicate that binding to the inositol phosphate head group is necessary but may not be sufficient for membrane localization of the PLCδ_1PH-GFP fusion protein, and motifs located within the C-terminal half of the PH domain provide auxiliary contacts with additional membrane components.

Phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$),[1] a minor phospholipid component of the plasma membrane, is a key regulator of several cellular processes. $PI(4,5)P_2$ is a precursor of important second messengers, such as the diffusible $InsP_3$, which regulates Ca^{2+} release from intracellular Ca^{2+} stores, and the protein kinase C activator, diacylglycerol (1, 2). $PI(4,5)P_2$ is also phosphorylated by class I PI 3-kinases to form $PI(3,4,5)P_3$, which controls membrane recruitment and the functions of several important signaling proteins (3). $PI(4,5)P_2$ itself is a regulator of a great variety of target molecules, including ion channels (4, 5) and several proteins that regulate actin polymerization and the cytoskeleton (see Ref. 6), providing a link between the plasma membrane and the cortical cytoskeleton (7). $PI(4,5)P_2$ has been implicated in several forms of membrane remodeling events, including the fusion of secretory vesicles with the plasma membrane (8), clathrin-mediated endocytosis (9–11), and membrane recovery by endocytosis during neurotransmitter release (12) (also see Ref. 13 for a review). Such diverse functions must rely upon interaction of the lipid with a large number of regulatory molecules. Most proteins that bind $PI(4,5)P_2$ contain a sequence composed of basic residues that provide electrostatic interaction with the phosphate groups of the inositol ring (6). Recent advances revealing the three-dimensional structures of several protein motifs that bind phosphoinositides offer a deeper insight into their molecular recognition (14). One of the first protein modules capable of binding membrane $PI(4,5)P_2$ was identified in pleckstrin, the major protein kinase C substrate in platelets (15). Pleckstrin homology (PH) domains have since been described in a large number of signaling proteins, and they show remarkable specificity in recognizing various forms of inositides. It is generally believed that at least one of the functions of PH domains is to provide proper localization of proteins via interactions with inositol phospholipids. However, some PH domains have also been shown to bind proteins (16–18), and their protein binding together with lipid binding may act in concert to regulate the localization and/or the function of those proteins. In addition to PH domains, additional motifs, such as FYVE domains (19, 20) and the PX domains (21, 22), have been shown to bind phosphoinositides, namely the lipid product of the class III PI 3-kinases.

Because recognition of inositol lipids by PH domains is based on the specific interaction of key surface residues with the phosphate groups of the inositide head group (23), it is not

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[1] The abbreviations used are: $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; Ang II, angiotensin II; AT_{1A}, type IA angiotensin II receptor; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_4$, inositol 1,3,4,5-tetrakisphosphate; $InsP_6$, inositol hexakisphosphate, PLCδ_1, phospholipase C δ_1; PH domain, pleckstrin homology domain; GFP, green fluorescent protein; YFP, yellow fluorescent protein; PIP, phosphatidylinositol-phosphate; NTA, nitrilotriacetic acid; FRET, fluorescence resonance energy transfer.

surprising that many (although not all) PH domains also bind to the soluble inositol phosphate counterpart of their lipid binding partner, a feature that is widely utilized to study their binding properties (24). It is also known that hydrophobic interactions also contribute to the membrane localization of PH domains (25). In the case of several PH domains it is not quite clear whether membrane localization via lipid binding or binding to soluble inositol phosphates is more important for their regulation. For example, the membrane localization of PLCδ_1 via its PH domain and hence its activity is regulated by $InsP_3$ levels (26). Rapid and large $InsP_3$ increases can translocate a PLCδ_1PH-GFP fusion protein from the membrane to the cytosol (27, 28). Also, the PH domain of the $InsP_4$-binding protein, $GAP1^{IP4BP}$, is believed to be the target of $InsP_4$ (29), but it also anchors the protein to the membrane via phosphoinositide binding (30). These data prompted us to investigate the question of whether binding to the inositol phosphate head group (such as to $InsP_3$) or to the lipid, $PI(4,5)P_2$, is sufficient to localize a PH domain to the cell membrane. While creating protein domains with high $InsP_3$ binding affinity, we also explored the possibility whether such molecules could be used as research tools to alter Ca^{2+} signaling by sequestering $InsP_3$.

Here we used the PH domains of PLCδ_1 and the recently cloned PLC-like protein, p130, as well as the $InsP_3$ binding domain of the type I $InsP_3$ receptor to demonstrate that high affinity binding to $InsP_3$ or even to $PI(4,5)P_2$ is not sufficient to recruit these proteins to the plasma membrane. We also propose that regions other than those participating in recognition of the inositide head group are important for the membrane recruitment of the PLCδ_1 PH domain and perhaps of other similar protein modules.

EXPERIMENTAL PROCEDURES

Materials—Angiotensin II (human) was purchased from Peninsula Laboratories. Ionomycin, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, $InsP_3$, $InsP_4$, and $InsP_6$ were obtained from Calbiochem. Phosphatidylinositol, phosphatidylserine, and phosphatidylcholine were all from Sigma. diC_8-$PI(4,5)P_2$ and the PIP strips were purchased from Echelon (Salt Lake City, UT). *myo*-[^{3}H]Inositol (68 Ci/mmol) and [^{3}H]$InsP_3$ (48 Ci/mmol) were from Amersham Biosciences. All other chemicals were of high performance liquid chromatography or analytical grade.

DNA Constructs—The PH domain of human PLC-δ_1 (GenBank accession number U09117) (residues 1–170), its mutant R40L, as well as its truncated version (residues 1–135) has been described previously (31). The PH domain of the p130 protein (GenBank accession number D45920) (residues 95–233) was amplified from rat brain cDNA (Quickclone, CLONTECH, Palo Alto, CA) using the primers 5′-ACAGA ATTCA CCATG GTGTC TTTCA GCAGC ATGCC ATC-3′ and 5′-CAGTG GATCC ATAAA GTCAA GTGGT TGCTT ACTGC GAG-3′. After digestion with *Eco*RI and *Bam*HI the product was ligated into the pEGFP-N1 plasmid (CLONTECH) digested with the same enzymes. p130PH was also cloned into the pEGFP-C1 plasmid (CLONTECH) after amplification with the following primer pairs: 5′-CTTCCTCGAGT GTCTT TCAGC AGCAT GCCA-3′ and 5′-TAAGA ATTCA CATAA AGTCA AGTGG TTGCT-3′ and digestion with *Xho*I and *Eco*RI. Chimeric PH domains were created by introducing an *Eco*RV site to PLCδ_1PH-GFP at residues 71–72 (silent) or at 111–112 (L110I) and to p130PH-GFP at residues 163–164 (A163D) or 204–205 (L204I) for swapping the C-terminal sequences (starting from either the β5 or the β7 strands, respectively) between the two molecules. These conservative mutations alone did not alter the cellular localization of the proteins. The short 8-amino acid loop located between the β6 and β7 strands of PLCδ_1PH-GFP was substituted for the same segment in p130PH-GFP with the following primers: 5′-ctc ttc aag gac caa cgc aac act ctg gac cta gttgc-3′ and 5′-gtc cag agt gtt gcg ttg gtc ctt gaa gag tat gga ga-3′. All mutations were verified with dideoxy sequencing.

The $InsP_3$ binding domain (residues 1–610) of the human type I $InsP_3$ receptor (GenBank accession number D26070) was amplified from human brain cDNA (Quickclone, CLONTECH) with the following primer pairs: 5′-GCAAC AGAGT GCCTG ACCCA GGTCA G-3′ and 5′-CTTTC GCACC AGGCT GACAA ATGTG TC-3′. The PCR product was subcloned into the pGEM-Easy T/A cloning plasmid (Promega, Madison, WI). After sequencing, two clones were identified, one containing (SII+) and the other lacking (SII−), the linker region located between the two domains involved in $InsP_3$ binding (32). By using the SII+ splice variant as template, the $InsP_3$ binding domain (residues 224–605) was amplified with nested primers containing *Xho*I and *Eco*RI restriction sites, and the PCR product was subcloned into the pEGFP-C1 vector. All constructs were sequenced with dideoxy sequencing. The integrity and expression levels of the fusion proteins were assessed by Western blot analysis from cells lysates prepared from COS-7 or NIH 3T3 cells transfected with the constructs, using a polyclonal antibody against GFP (CLONTECH).

Transfection of Cells for Confocal Microscopy—Cells were plated onto polylysine-coated 30-mm diameter circular glass coverslips at a density of 5×10^4 cells/35-mm dish and cultured for 3 days before transfection with plasmid DNAs (1 μg/ml) using the LipofectAMINE reagent (10 μg/ml, Invitrogen) and Opti-MEM (Invitrogen). One day after transfection, cells were washed twice with a modified Krebs-Ringer buffer (120 mM NaCl, 4.7 mM KCl, 1.2 mM $CaCl_2$, 0.7 mM $MgSO_4$, 10 mM glucose, Na-Hepes 10 mM, pH 7.4), and the coverslip was placed into a chamber that was mounted on a heated stage with the medium temperature kept at 33 °C. Cells were incubated in 1 ml of the Krebs-Ringer buffer, and stimuli were added in 0.5 ml of prewarmed buffer after removing 0.5 ml of medium from the cells. Cells were examined in an inverted microscope under a 40× oil immersion objective (Nikon) and a Bio-Rad laser confocal microscope system (MRC-1024) with the Lasersharp acquisition software (Bio-Rad).

Recombinant Proteins and InsPx Binding Assays—For bacterial expression of the GFP-fused protein domains, the coding sequences were amplified from the T/A cloning plasmids (see above) and were subcloned into the pET-23b bacterial expression vector (Novagen) using the *Xho*I/*Eco*RI (for p130PH-GFP and PLCd$_1$PH-GFP) and the *Nco*I/*Eco*RI restriction sites (for the GFP-$InsP_3$R-(224–605) construct). The resulting plasmids were used to transform the BL-21-DE3 strain of *Escherichia coli* (Novagen). Bacterial cells were grown to A_{600} 0.6 at 37 °C and induced with 300 μM isopropyl-1-thio-β-D-galactopyranoside at 18–20 °C for 7 h. Bacterial lysates were prepared by sonication in lysis buffer (20 mM NaCl and 20 mM Tris, pH 8.0) followed by centrifugation at $10,000 \times g$ for 30 min at 4 °C. The supernatant was incubated with Ni^{2+}-NTA-agarose beads (Qiagen) in the presence of 5 mM imidazole for 1 h at 4 °C. The beads were washed three times with lysis buffer, and the recombinant proteins were eluted with the same buffer containing 1 M imidazole. Protein samples were concentrated and stored in phosphate-buffered saline containing 5 mM dithiothreitol and 20% glycerol at −20 °C. The concentration of recombinant proteins was assessed by quantifying the bands of Coomassie Blue-stained SDS gel containing the recombinant proteins and bovine serum albumin as a standard.

The incubation buffer of the *in vitro* $InsP_3$ binding assay contained 50 mM Na-Hepes, pH 7.4, 50 mM KCl, 0.5 mM $MgCl_2$, and 0.01 mM $CaCl_2$. About 0.2 μg of soluble recombinant proteins was incubated in 50 μl of this buffer with 0.74 kBq (0.5 nM) [^{3}H]$Ins(1,4,5)P_3$ and the various unlabeled inositol phosphates or short side-chained inositol lipids for 10 min on ice. The binding reaction was terminated by adding 5 μl of human γ-globulin (10 mg/ml) and 50 μl of polyethylene glycol 6000 (30%) (24). Tubes were left on ice for 5 min and were centrifuged at $10,000 \times g$ for 10 min. The precipitates were dissolved in 0.1 ml of 2% SDS, and the radioactivity was counted in a liquid scintillation counter. Binding assays were also performed on proteins still bound to Ni^{2+}-NTA beads, where separation of the protein-bound ligand (on the beads) from unbound ligand was achieved by adding 100 μl of a mixture of bis(3,5,5-trimethylhexyl)-phthalate and dimethyl phthalate (density, 1.010 g/ml) and centrifugation at $15,000 \times g$ at 4 °C for 5 min.

For binding of recombinant proteins to lipids on PIP strip membranes (33), 100 pmol of recombinant protein was incubated in 5 ml of binding buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 3% bovine serum albumin (lipid-free), 2 mM sodium pyrophosphate, and 0.1% Tween 20) overnight at 4 °C, after blocking the strips with the same buffer for 90 min at room temperature. After washing, GFP was visualized by Western blotting using the polyclonal anti-GFP antibody from CLONTECH essentially as described previously (34).

Analysis of Cytosolic Ca^{2+} Responses in Individual COS-7 Cells—COS-7 cells were plated onto polylysine-coated coverslips and cultured for 3 days prior to experiments. Cells were transfected with plasmid DNAs (see above) and were loaded with the fluorescent Ca^{2+} indicator, fura-2, 24–48 h after transfection. Loading was achieved by incubating cells with 5 μM fura-2/AM for 25–30 min at room temperature in a medium containing 121 mM NaCl, 5 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM Na-Hepes, pH 7.4, and 2% bovine serum albumin supplemented with 0.003%

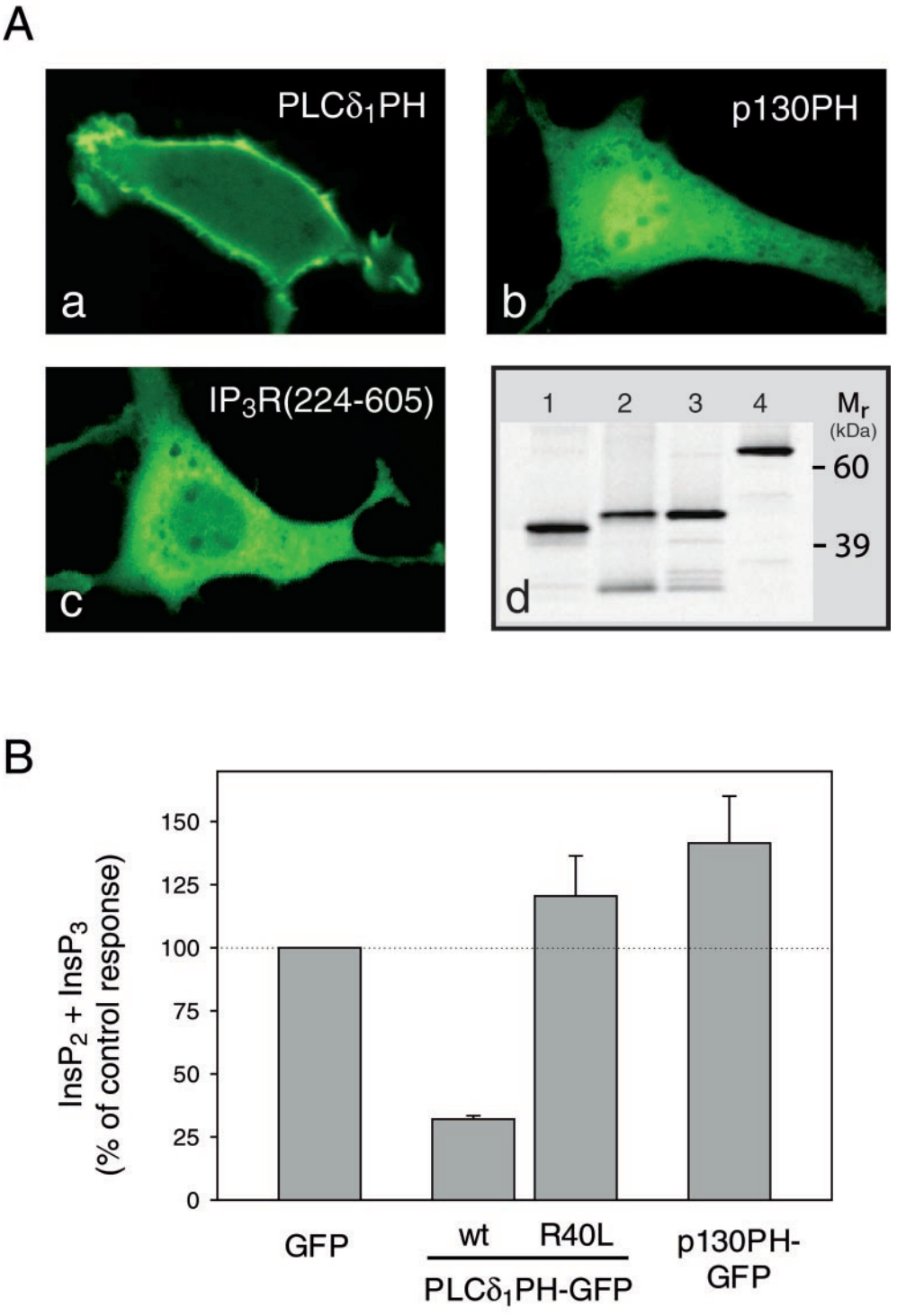


FIG. 1. **Cellular localization of protein modules that bind $InsP_3$ (*A*) and their effect on angiotensin II-stimulated InsP production (*B*).** The PH domains of $PLC\delta_1$ or of the PLC-related protein, p130, were fused to the N terminus of enhanced GFP, whereas the $InsP_3$ binding domain of the type I $InsP_3$ receptor was fused to the C terminus of enhanced GFP. The resulting constructs were transfected into NIH 3T3 cells, and the cells were examined by confocal microscopy. The same constructs, tagged with a His_6 tag at their C terminus, were also created for expression in *E. coli*, and the expressed proteins were purified on Ni^{2+}-NTA columns (see "Experimental Procedures" for details). The recombinant fluorescent proteins retained their fluorescence after SDS-PAGE (without boiling) and could be analyzed in a PhosphorImager (*lanes 1–4* represent p130PH-GFP, $PLC\delta_1$PH-GFP, R40L$PLC\delta_1$PH-GFP, and GFP-IP_3R-(224–605), respectively). *B*, inositol phosphate production was assessed in [^{3}H]inositol-labeled COS-7 cells transiently expressing the AT_{1A} angiotensin receptor and the indicated GFP proteins. Labeled cells were stimulated with angiotensin II for 30 min after a 30-min preincubation with 10 mM LiCl (37). $InsP_3$ and $InsP_2$ fractions obtained from Dowex AG 1-X8 columns were combined. Values are expressed as percent of the control response (2–3-fold), and the results are the means ± S.E. of 4–5 experiments performed in duplicate.

pluronic acid and sulfinpyrazone (200 μM). After loading, cells were washed with the same medium containing 0.25% bovine serum albumin supplemented with sulfinpyrazone but without fura-2/AM. Coverslips were then mounted on the thermostated stage (35 °C) of an Olympus IX70 inverted microscope coupled to a high quantum efficiency cooled CCD camera driven by a customized computer program that also controlled a scanning monochromator (DeltaRAM, PTI) to select multiple excitation wavelengths (35). Fura-2 fluorescence was measured (340

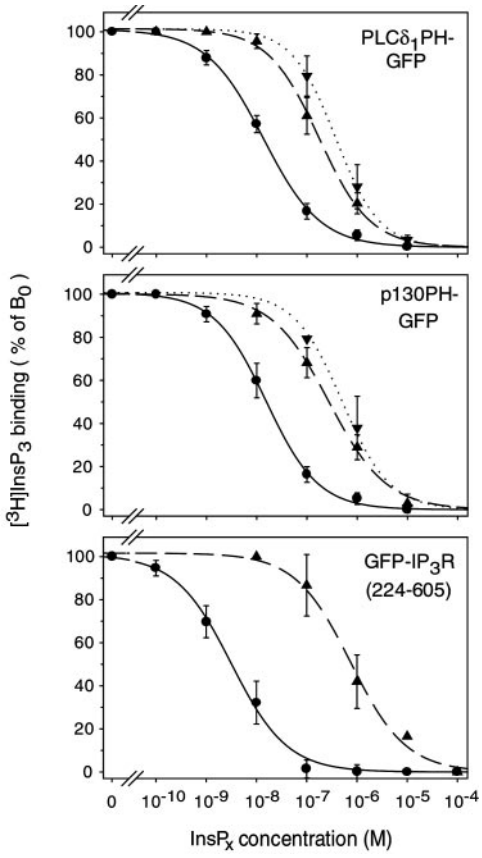


FIG. 2. **Inositol phosphate binding characteristics of recombinant $InsP_3$ binding domains fused to GFP.** Proteins were expressed in *E. coli* and were purified by Ni^{2+}-NTA chromatography (see Fig. 1, *panel d*). Binding assays were performed using [^{3}H]$InsP_3$ in the presence of the indicated concentrations of the respective unlabeled ligand ($InsP_3$, *filled circles*), ($InsP_4$, *filled triangles*), and ($InsP_6$, *inverted filled triangles*) as detailed under "Experimental Procedures." The specific binding (100%) was between 3000 and 6000 cpm in these experiments. Means ± S.E. of 4–5 experiments performed in duplicate are shown.

and 380 nm excitations) simultaneously with enhanced GFP fluorescence (490 nm excitation) using a 510-nm long pass dichroic mirror and a 520-nm long pass emission filter. Cells were stimulated with 50 μM ATP, which evokes a Ca^{2+} signal via the endogenous P_{2y} receptor of COS-7 cells. Fluorescence signals were calculated for the total area of individual cells, and the background fluorescence obtained over cell-free regions of each image was subtracted prior to calculation of the fluorescence ratios. Recordings obtained from all transfected cells on the field were averaged for comparison in each experiment. Experiments were performed on 3–4 separate cell transfections.

Analysis of Inositol Phosphates—Inositol phosphates were analyzed from COS-7 cells transfected with cDNA encoding the rat AT_{1A} angiotensin receptor, together with selected GFP-PH domain fusion constructs as described previously (36). One day after transfection, cells were labeled with *myo*-[^{3}H]inositol for 24 h. After washing with inositol-free M199 and 30 min of preincubation in the presence of 10 mM LiCl, cells were stimulated with Ang II (1 μM) for 30 min. Reactions were then terminated, and ^{3}H-labeled inositol phosphates were extracted and separated by Dowex minicolumns as described previously (37).

Measurements of Binding to PI(4,5)P_2 Containing Lipid Vesicles—Phospholipid binding was performed with mixed lipid vesicles. 110 μg of PI(4,5)P_2 (Roche Molecular Biochemicals) and 1.4 mg of phosphatidylethanolamine (bovine liver; Avanti) were mixed and dried under a nitrogen stream followed by high vacuum, and the dried mixtures were suspended to a final total lipid concentration of 2 mM in 20 mM Hepes, pH 7.2, 100 mM NaCl, 2 mM EGTA, 0.1 μg/ml bovine serum albumin by bath sonication. 5 μl of the purified GFP fusion protein (1 μg) and 5 μl of inositol 1,4,5-trisphosphate stock solution were added to 90 μl of phospholipid vesicles. As a precaution, proteins were subjected to ultracentrifugation (85,000 × *g* for 20 min at 4 °C) prior to the assays to remove possible protein aggregates, although protein preparations were used freshly when they had no significant aggregation. The reaction mixture was incubated at 30 °C for 10 min followed by ultracentrifugation at 85,000 rpm for 20 min at 4 °C. The 100-μl supernatant was mixed with 30 μl of 5× Laemmli buffer, and the pellet was resuspended in 100 μl of incubation buffer followed by the addition of 30 μl of 5× Laemmli buffer. After vortexing, 40 μl of each fraction was loaded onto a 12% Tris glycine gel without boiling and separated by SDS-PAGE at 4 °C. After electrophoresis, gels were analyzed in a Storm 860 (Amersham Biosciences) PhosphorImager using blue fluorescence screening for quantitation of the GFP fusion protein band on the gel. Western blot analysis was also performed on parallel samples using the purified polyclonal antibody against GFP (CLONTECH).

FRET Measurements—To have a quantitative measure of membrane localization of the various constructs, the CFP and YFP variants of all fusion proteins have been created. These were co-transfected into COS-7 cells that were cultured in 10-cm culture dishes. One day after transfection, cells were removed from the dishes by mild trypsinization, washed, and centrifuged. Cells (about 3–5 million) were then resuspended in 2 ml of the Krebs-Ringer solution described above and placed in the thermostated cuvette holder of a fluorescence spectrophotometer used for ratiometric Ca^{2+} measurements. Recordings were made using an excitation of 425 nm and calculating a ratio from the emissions detected at 525 and 475 nm (20 nm bandwidth each). Ionomycin (10 μM) was used to activate endogenous phospholipase C to hydrolyze the phospholipids, and the decrease in the 525:475 ratio was taken as an index of translocation of the domains from the membrane to the cytosol (see Refs. 28 and 31 for details concerning the FRET approach and ionomycin manipulation, respectively).

RESULTS

Cellular Distribution of Protein Domains Capable of Binding Ins(1,4,5)P_3—The PH domain of PLCδ_1 binds PI(4,5)P_2 of the plasma membrane and has been used as a tool to visualize changes in the level of this lipid in living cells in the form of a GFP fusion protein (31, 38). However, it has also been noted that PLCδ_1PH-GFP does not bind to other intracellular membranes despite extensive biochemical and immunocytochemical (using anti-PI(4,5)P_2 antibodies) evidence that PI(4,5)P_2 is also present in other cellular membranes such as the Golgi and the nucleus (39–41). These observations prompted us to investigate whether additional PH domains found in related proteins could recognize PI(4,5)P_2 in other cellular compartments. During these efforts we isolated the PH domain (residues 95–233) of the recently described PLC-like protein, p130, which was initially described as an $InsP_3$-binding protein with binding features very similar to those of PLCδ_1 (42). We also isolated the $InsP_3$-binding region of the type I $InsP_3$ receptor (residues 224–605) to examine whether its high affinity binding to $InsP_3$ also allows it to bind to membrane PI(4,5)P_2.

As shown in Fig. 1*A*, neither p130PH-GFP nor GFP-IP_3R-(224–605) showed membrane localization when expressed in NIH 3T3 cells. This was in contrast to the well documented membrane localization of PLCδ_1PH-GFP. Expression of the constructs in a variety of mammalian cells (COS-7, Madin-Darby canine kidney, and HEK-293) yielded no membrane localization of either of the two proteins (not shown). The lack of binding of p130PH-GFP to membrane PI(4,5)P_2 in COS-7 cells was also confirmed by analysis of inositol phosphate production in response to Ang II stimulation. As documented previously, expression of PLCδ_1PH-GFP interferes with PLC activation, a property that closely correlates with the ability of the protein to bind PI(4,5)P_2 of the plasma membrane (31). As shown in Fig. 1*B*, expression of p130PH-GFP had no inhibitory effect on the Ang II-stimulated InsP response, and if anything, it slightly enhanced the response.

$InsP_3$ and PI(4,5)P_2 Binding to Recombinant Protein Domains—To investigate the inositol lipid and inositol phosphate-binding properties of these proteins, we created the same constructs for bacterial expression. Proteins were purified on Ni^{2+} columns, and *in vitro* binding assays were performed using [^{3}H]$InsP_3$ as the tracer. As shown in Fig. 2 and Table I, p130PH-GFP was able to bind $InsP_3$ with an affinity that was

TABLE I

IC_{50} values (in nM) for the displacement of [^{3}H]Ins(1,4,5)P_3 from the various GFP-tagged Ins(1,4,5)P_3 binding domains and for the ability of Ins(1,4,5)P_3 to inhibit their association with PI(4,5)P_2-containing lipid vesicles

Means ± S.E. are shown with the number of observations in parentheses. ND, not determined.

	PLCδ_1PH-GFP	p130PH-GFP	GFP-IP$_3$R (224–605)
[^{3}H]Ins(1,4,5)P_3 binding displaced by			
Ins(1,4,5)P_3	17 ± 0.4 (8)	22 ± 8 (9)	4 ± 2 (6)
Ins(1,3,4,5)P_4	219 ± 60 (4)	308 ± 88 (3)	868 ± 496 (2)
InsP_6	528 ± 276 (4)	764 ± 422 (3)	ND
Di(C_8)PI(4,5)P_2	91 ± 24 (4)	91 ± 17 (4)	ND
Binding to PI(4,5)P_2 displaced by			
Ins(1,4,5)P_3	1000 ± <50 (6)	300 ± <15 (6)	ND

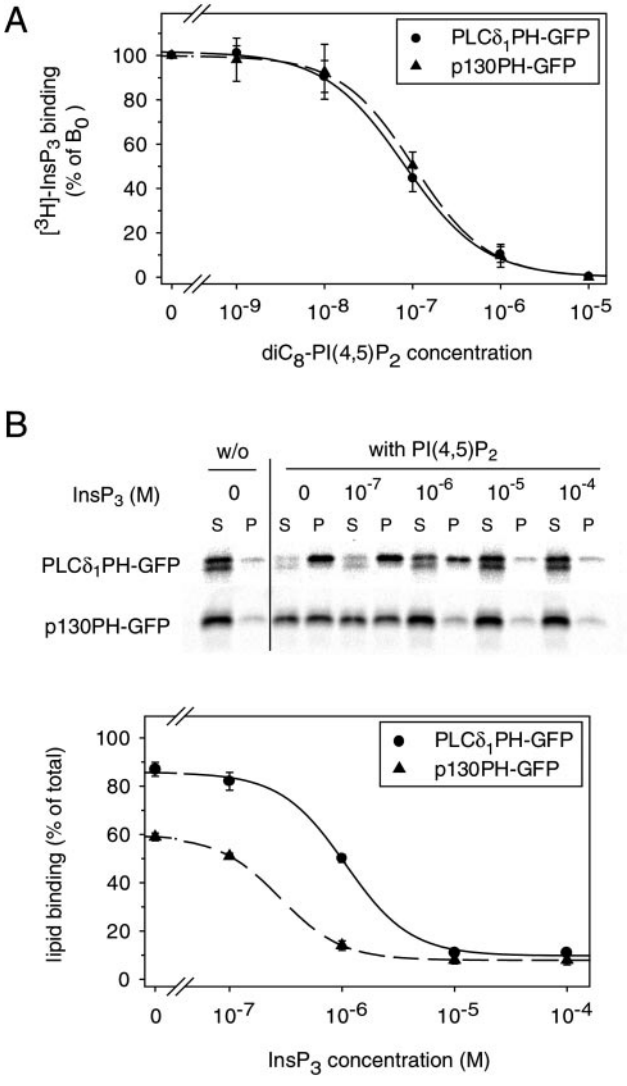


FIG. 3. **Inositol lipid binding of recombinant InsP_3 binding domains fused to GFP.** Proteins were expressed in *E. coli* and purified by Ni^{2+}-NTA chromatography. *A,* [^{3}H]InsP_3 binding was examined in the presence of increasing concentrations of water-soluble forms of PI(4,5)P_2 (*diC_8-PI(4,5)P_2*). *B,* binding of recombinant proteins to lipid vesicles containing PI(4,5)P_2 in the presence of increasing concentrations of InsP_3. *S* and *P* represent the soluble (unbound) and pellet-associated (bound) GFP fusion protein, respectively, analyzed by a PhosphorImager after SDS-PAGE. *Lower panel* shows the summary of six similar experiments (mean ± S.E.).

indistinguishable from that of PLCδ_1PH-GFP. Mutant forms of either proteins (R40L-PLCδ_1PH-GFP and R134L-p130PH-GFP) showed no [^{3}H]InsP_3 binding, indicating that InsP_3 binding was specific to the recombinant wild-type proteins and not to any impurities present in the preparations (not shown). The GFP-IP$_3$R-(224–605) protein showed higher affinity to InsP_3 than either of the two PH domains, and its affinity was comparable with that of the intact InsP_3 receptor or with a similar binding domain produced in *E. coli* as a GST fusion protein (43). All three proteins discriminated between InsP_3 and InsP_4, but the receptor was significantly more selective in this respect. InsP_6 was almost as effective as InsP_4 in displacing [^{3}H]InsP_3 from both p130PH-GFP and PLCδ_1PH-GFP, indicating that the inositol phosphate binding characteristics of the two PH domains are very similar. These findings suggested that the inability of p130PH-GFP or GFP-IP$_3$R-(224–605) to localize to cellular membranes in intact cells is not due to their inability to recognize the inositol phosphate head group.

To investigate the relative affinities of these proteins to inositol lipids rather than inositol phosphates, we used three different approaches. First, a water soluble, short side-chain analogue of PI(4,5)P_2 (diC_8-PI(4,5)P_2) was used as a competitor in the [^{3}H]InsP_3 binding assays. No difference was found between the affinities of PLCδ_1PH-GFP and p130PH-GFP to diC_8-PI(4,5)P_2 in these experiments (Fig. 3*A* and Table I). Both proteins showed significantly lower affinities to the soluble lipid derivatives than to InsP_3. Second, the binding of PLCδ_1PH-GFP and p130PH-GFP to lipid vesicles containing PI(4,5)P_2 was compared. Both proteins were able to bind to such vesicles in this assay; however, a larger fraction of the PLCδ_1PH-GFP was found vesicle-bound, and higher concentrations of InsP_3 were needed to displace it from the vesicles than in the case of the p130PH-GFP (Fig. 3*B*). The R40L mutant of PLCδ_1PH-GFP showed no specific localization to lipid vesicles that would be displaced by InsP_3 (data not shown). A third approach to compare the lipid binding of these proteins was to study the binding of the recombinant proteins to lipids spotted on nitrocellulose membranes as described previously (33, 34). As shown in Fig. 4, PLCδ_1PH-GFP and p130PH-GFP showed very similar inositol-lipid binding specificity and p130PH-GFP only a slightly lower affinity to PI(4,5)P_2 based on these assays. The R40L mutant PLCδ_1PH-GFP showed no binding under the same conditions (Fig. 4).

Protein Domains That Bind Ins(1,4,5)P_3 Can Interfere with Agonist-induced Ca^{2+} Signaling—Whereas the *in vitro* binding data clearly showed that the proteins made in bacteria are able to bind InsP_3 and PI(4,5)P_2 *in vitro*, we also wanted to examine whether their InsP_3 binding is manifested in intact cells. For this, we used COS-7 cells in which the various GFP fusion proteins were expressed. Expression of a protein that binds InsP_3 with high enough affinity is expected to buffer InsP_3 increases with consequences on Ca^{2+} signaling when cells are stimulated by an agonist that stimulates phospholipase C. To test this, we used ATP to stimulate the endogenous P_{2y} receptors of COS-7 cells expressing the various constructs. As shown in Fig. 5, expression of both p130PH-GFP and GFP-IP$_3$R-(224–605), but not GFP alone, had a marked effect on the ATP-evoked Ca^{2+} signal in the COS-7 cells. The most prominent

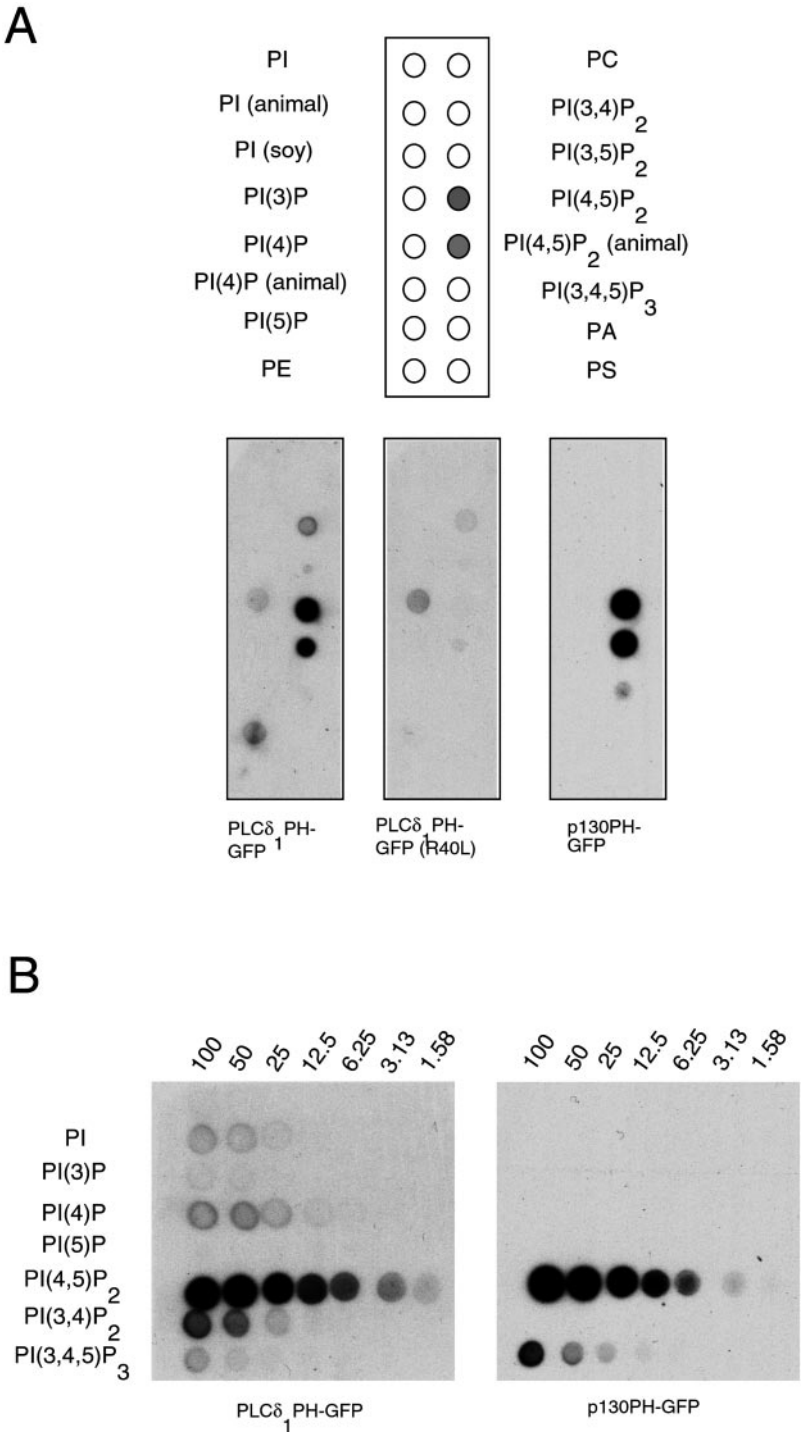
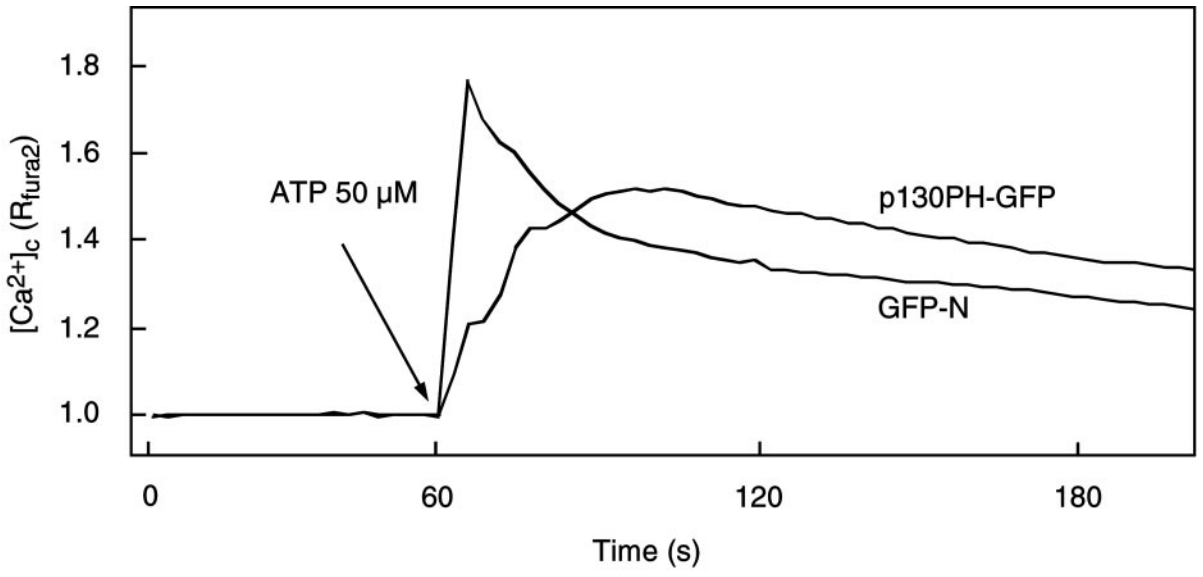


FIG. 4. **Binding of recombinant PLCδ_1PH-GFP and p130PH-GFP to various phosphoinositides.** Proteins were expressed in *E. coli* and purified by Ni^{2+}-NTA chromatography. PIP strips were incubated with 100 pmol of the recombinant proteins overnight at 4 °C as detailed under "Experimental Procedures." After washing, GFP was visualized by Western blotting using the polyclonal anti-GFP antibody from CLONTECH essentially as described previously (34). *A* shows binding of the recombinant proteins to the various lipids (300 pmol/spot) and the specificity of both PH domains to PI(4,5)P_2. *B* shows binding of the recombinant proteins to decreasing amounts (in pmol/spot) of the lipids, revealing very similar affinities of PLCδ_1PH-GFP and p130PH-GFP to PI(4,5)P_2.

effect was an increase in the lag time (the time that is required to reach half-maximal Ca^{2+} response) from the time of ATP addition. Because expression of p130PH-GFP did not inhibit agonist-induced PLC activation (and presumably $InsP_3$ production, see above), the increased lag time was consistent with the buffering of $InsP_3$ by the expressed domains. Under these conditions, after stimulation of the P_{2y} receptors by ATP, it takes a longer time to reach the level of $InsP_3$ where the coordinated action of $InsP_3$ and Ca^{2+} triggers Ca^{2+} release (44). This assay was sensitive enough to detect the $InsP_3$ affinity difference between the constructs, because GFP-IP_3R-(224–605) had a significantly bigger effect on the lag time than p130PH-GFP (Fig. 5, *lower panel*).

Analysis of Cellular Distribution of Chimeric Proteins Constructed from the PH Domains of PLCδ_1 and p130—Together these data indicated that although p130PH is capable of binding $InsP_3$ as well as PI(4,5)P_2, this binding is not sufficient to recruit the protein to the plasma membrane. Given the level of similarity between the two amino acid sequences (Fig. 6), we decided to create chimeras from the two proteins to investigate which part of the PLCδ_1PH accounts for its ability to localize to the plasma membrane. Because most of the contacts with $InsP_3$ in PLCδ_1PH are found within the N-terminal part of the molecule (containing the β1–β4 sheets) (45) (see also Fig. 6), first we designed a chimera in which the N-terminal halves of the PH domains were exchanged between the two proteins. These



Transfection	Lag time (s)	Number fo cells	F_{GFP} (a.u.)
no GFP	4.4 ± 0.2	329	-
GFP-N	3.1 ± 0.2	55	4770 ± 340
GFP-$IP_3R_{224-605}$	26.6 ± 4.3	40	2235 ± 282
p130PH-GFP	14.5 ± 2.3	47	4007 ± 383

FIG. 5. **Inhibition of agonist-induced Ca^{2+} signaling by expressed $InsP_3$-binding modules fused to GFP.** COS-7 cells were transfected with the indicated GFP fusion constructs, and their cytosolic Ca^{2+} responses were analyzed by ratiometric Ca^{2+} imaging using fura2. The lag time of the Ca^{2+} response was defined as the time it took for a cell to reach half-maximum of its Ca^{2+} peak after addition of 50 μM ATP. *Upper panel* shows the average Ca^{2+} signals calculated from the number of recordings obtained from transfected cells expressing either p130PH-GFP or GFP. *Lower panel* contains the average lag times calculated for the cells expressing the indicated fusion protein. The number of cells recorded within the individual groups and their average fluorescence are also indicated.

chimeras were then expressed in either NIH 3T3 or COS-7 cells, and their cellular distribution was observed. No plasma membrane localization was observed with the chimera containing the N-terminal $InsP_3$-binding half of PLCδ_1PH fused to the C terminus of the p130PH-GFP (PLCδ1-(1–71)/p130-(166–233)-GFP) (Fig. 7). In contrast, the inverse chimera (p130-(95–164)/PLCδ1-(71–170)-GFP) showed clear membrane localization, although this was significantly less than that of the original PLCδ_1PH-GFP (Fig. 7). In both cases the expressed chimeras showed some intracellular "precipitates" in a number of cells, especially in those expressing high amounts of the protein. In our experience this indicates limited solubility or folding problems of the proteins. Similar phenomena were observed with other fluorescent proteins of limited solubility, for example with the PLCδ_1PH-BFP protein, but in the latter case the membrane localization of the protein could still be observed (not shown).

These data suggested that the C-terminal half of PLCδ_1PH contains additional determinant(s) for membrane localization and also showed that the $InsP_3$-binding region of p130PH can substitute to some extent for the corresponding part of PLCδ_1PH to support membrane localization. Therefore, additional chimeras were created in which the C-terminal helices that follow the β7 strands were exchanged between the two PH domains. These experiments showed that a p130PH-GFP containing the C-terminal helix of PLCδ_1PH still failed to localize to the membrane. Surprisingly, the PLCδ_1PH domain with the C-terminal helix of p130PH showed no membrane association (not shown). Because the PLCδ_1PH domain used in these experiments was 35 amino acids longer at its C terminus than p130PH, we examined whether this extra stretch of amino acids makes a difference in membrane targeting. Truncation of PLCδ_1PH to the length corresponding to that of our p130PH construct (PLCδ_1PH-(1–135), which still contains the full PH domain), showed membrane localization comparable with that of the longer form (not shown). From these data we concluded that the C-terminal helix contributes to intra- or intermolecular interaction(s) that contribute to membrane localization. Next we created a p130PH-GFP in which the short loop between the β6 and β7 strands of PLCδ_1PH was inserted in place of the corresponding region in the p130PH wild-type sequence. This loop is in a position that could come in contact with the inositide head group or some other component of the membrane (Fig. 6). As shown in Fig. 7, this protein chimera showed clearly

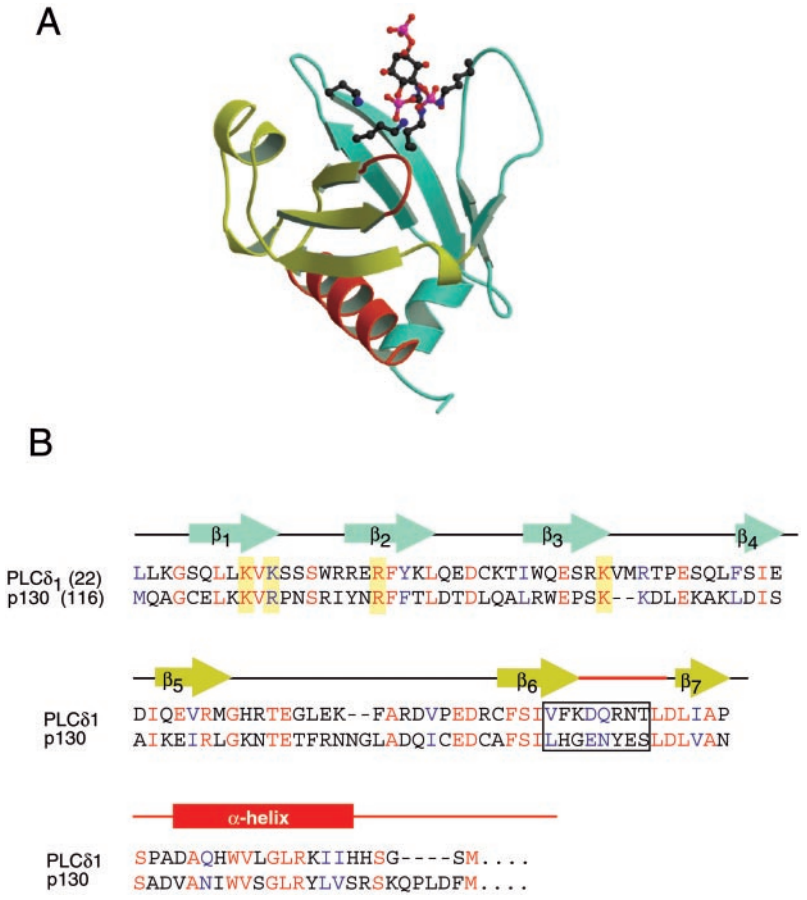


FIG. 6. **Sequence homology between the PH domains of PLCδ_1 and p130 and ribbon diagram showing the crystal structure of PLCδ_1PH.** *A* shows the ribbon diagram based on the crystal structure of PLCδ_1PH (23) generated by Molscript (62). The two different shades of *green* represent the two halves of the PH domain where the chimeric constructs were joined, and the *red* indicates the regions that were also exchanged between the two proteins. *B* shows the homology between the two primary sequences. *Green arrows* show the locations of the β-sheets. *Red* and *blue letters* indicate identical residues and conservative substitutions, respectively, and the basic residues coordinating the phosphates of InsP_3 are highlighted with *yellow*.

detectable membrane localization, although it was less pronounced than that of (p130-(95–164)/PLCδ1-(71–170)-GFP).

Localization of the chimeras to the plasma membrane was also assessed by FRET analysis. As shown previously, CFP- and YFP-tagged versions of the PH domains could transfer energy when found in molecular proximity at the membrane (28). We used this approach to assess the extent of membrane localization of the various chimeras. For this, COS-7 cells expressing both the CFP- and YFP-tagged PH domains were examined in suspension using a fluorescence spectrophotometer with excitation of 425 nm and emissions recorded at 475 and 525 nm to form the 525:475 ratio. In addition to FRET taking place in the plasma membrane, the absolute value of this ratio depends on several factors, including the relative expression levels of YFP-PH and CFP-PH and FRET that occurs in compartments other than the plasma membrane (*e.g.* in the nucleus). Because ionomycin induces a PLC-mediated breakdown of PI(4,5)P_2 in the plasma membrane and eliminates membrane localization, the amplitude of the ionomycin-induced decrease in the 525:475 ratio was taken as an index of FRET due to localization of the probes to membrane PI(4,5)P_2 (see Ref. 28 for details). These experiments showed that all of the domains that were able to localize to the plasma membrane (but none of those unable to bind) showed the characteristic decrease in 525:475 ratio in response to PLC activation, indicating their binding to the plasma membrane PI(4,5)P_2 pools prior to ionomycin addition (Fig. 7). The magnitude of changes upon Ca^{2+}-induced translocation showed a good correlation with the extent of PH domain localization reflected by the confocal images. It is noteworthy that the rate of re-association of the domains with the membranes during PI(4,5)P_2 resynthesis also mirrored the apparent affinity differences by which the various PH domains bound to the membrane.

DISCUSSION

There is increasing interest to understand the principles that govern regulation of many cellular functions by the highly compartmentalized changes in the levels of inositol phospholipids. An important aspect of this research is the characterization of structural features of the protein modules that interact with the inositide head group of inositides in the membrane. Several recent studies have analyzed the structural basis of the specific recognition of 3-phosphorylated inositides by PH domains and have pointed to the significance of the short variable loops between the β1–β2 and β3–β4 strands of such domains (46, 47). Similarly, the structural features determining the binding of FYVE domains (19, 20, 48) and PX domains to PI(3)P have been recently revealed (21, 49). To test specificity and compare relative affinities, it is convenient to analyze the binding properties of PH and other protein domains using soluble inositol phosphates and inositol lipids *in vitro*. However, it is equally important to test these interactions within the intact cell, because it is not possible to mimic in the test tube the exact conditions that prevail at cellular membranes.

Our interest in characterizing PH domains is originated from the need to understand and evaluate their usefulness in imaging inositide dynamics in living cells (31). The interpretation of the results of such imaging studies clearly depends on the molecular recognition properties of the protein domains. Several data suggest that various inositide binding domains interact only with a certain subset of the inositide pools (31). The restricted recognition of PI(4,5)P_2 by the PLCδ_1PH-GFP only within the plasma membrane prompted us to investigate other related PH domains for their PI(4,5)P_2 recognition properties. The PH domain of the p130 protein was one of our choices due to its similarity to PLCδ_1PH. The p130 protein has been orig-

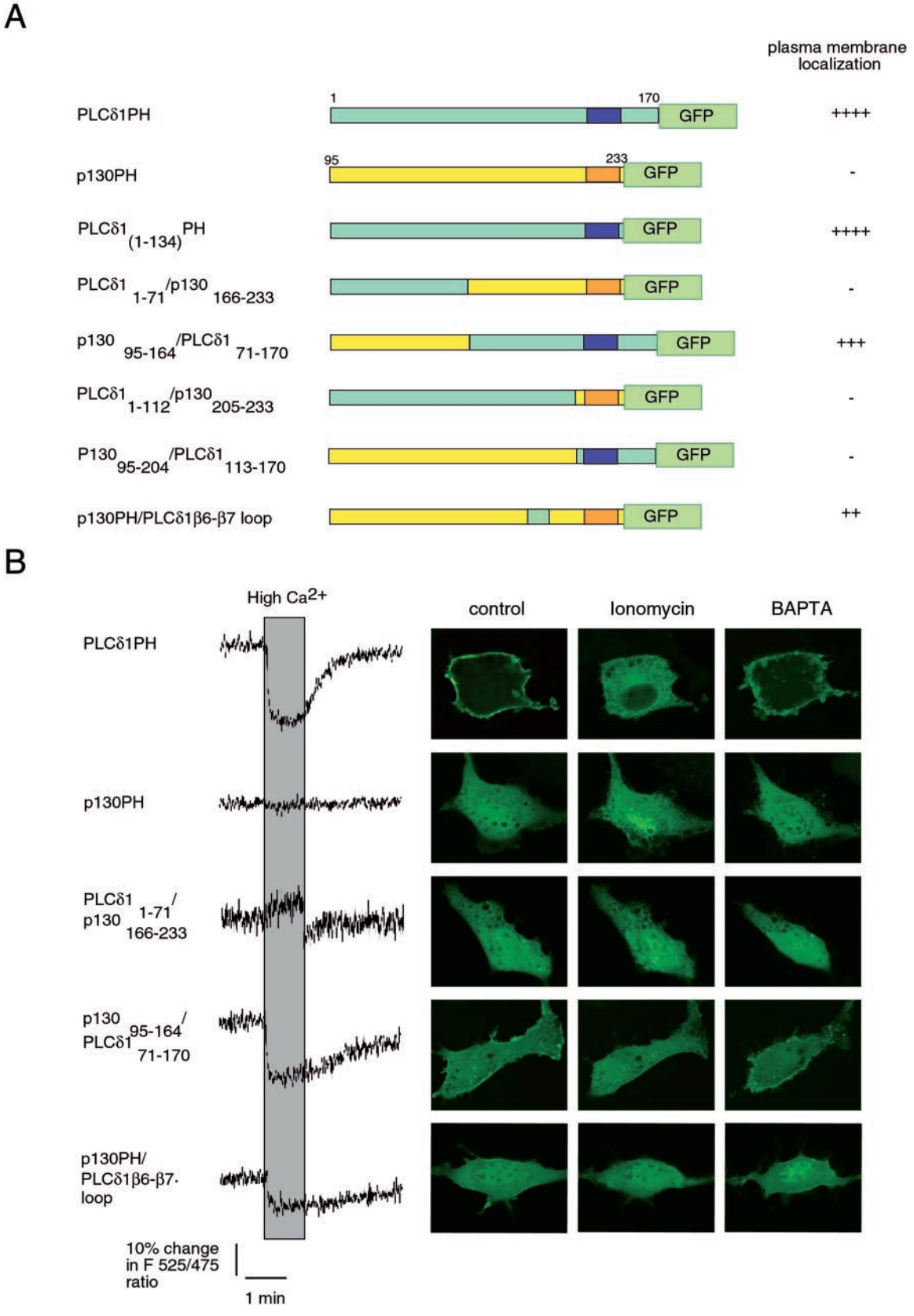


FIG. 7. **Cellular localization of various chimeras constructed from the PH domains of PLCδ_1 and p130.** *A* is a schematic diagram showing the various constructs and their plasma membrane localization scores based on confocal microscopy (in NIH 3T3 cells) and FRET analysis (COS-7 cells) of transfected cells. *B* shows representative confocal images of NIH 3T3 cells transfected with selected constructs and the FRET recordings obtained in COS-7 cell expressing the same proteins. Hydrolysis of PI(4,5)P_2 was initiated by the addition of 10 μM ionomycin in the presence of 1.8 mM external Ca^{2+} (labeled as high Ca^{2+}) and was terminated by the addition of 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (*BAPTA*). PI(4,5)P_2 hydrolysis is reflected in the redistribution of fluorescence from the membrane to the cytosol, whereas resynthesis of the lipid is paralleled by the relocalization of the fluorescent proteins to the membrane. Redistribution of the proteins due to Ca^{2+}-induced hydrolysis of membrane PI(4,5)P_2 was also assessed by FRET analysis (see "Experimental Procedures" and Ref. 28 for details).

inally described as an $InsP_3$-binding protein that was purified on an $InsP_3$ affinity matrix (42). Subsequent cloning of the protein revealed that it is a PLC homologue lacking PLC enzymatic activity and showing the greatest similarity to the yeast protein PLC1, the *Drosophila* NorpA, and mammalian PLCδ (50). Similarly to PLCδ$_1$, p130 possesses a PH domain that is responsible for the ability of the protein to bind $InsP_3$. Interestingly, despite its $InsP_3$ binding, the GFP-tagged p130 protein was found not to localize to membranes (51), and although wild-type p130 was reported to be associated with membranes when expressed in COS-7 cells, this association was not mediated by the PH domain (52). However, consistent with its $InsP_3$ binding, the ability of p130 to bind the second messenger $InsP_3$ has been demonstrated in intact cells because its overexpression altered both Ca^{2+} signaling and $InsP_3$ metabolism (51). The physiological function of this protein is still unknown, but it has been speculated that it might be important as a buffer of $InsP_3$ (51).

The present study clearly showed that $InsP_3$ binding even with high affinity, such as that of the type I $InsP_3$ receptor, is not sufficient to localize a protein to the membranes of intact cells through simple binding to the head group of $PI(4,5P)_2$. However, these experiments also show that even binding to the lipid $PI(4,5)P_2$ does not necessarily lead to membrane association. The small difference found between the $PI(4,5)P_2$-binding properties of isolated p130PH and PLCδ$_1$PH *in vitro* does not easily explain the striking difference between the abilities of the two domains to localize to the plasma membrane. The PH domain of PLCδ$_4$ was found to associate with cellular membranes with binding to $PI(4,5)P_2$ containing liposomes similar to that of p130PH-GFP.[2] Our data obtained with chimeras constructed from the PH domains of PLCδ$_1$ and p130 showed that the regions primarily responsible for $InsP_3$ binding (localized within the N-terminal half of the PH domain) are to some extent interchangeable between the two PH domains, consistent with their almost identical $InsP_3$ binding affinities. It is the C-terminal half of the PLCδ$_1$ PH domain that seems to contain additional feature(s) that are needed for membrane localization. Within this half of the molecule, we were able to identify the short loop between the β6–β7 strands of PLCδ$_1$PH as a sequence that aides membrane localization. However, the surprising loss of membrane localization of the PLCδ$_1$PH when containing the C-terminal helix of p130 also indicates important contributions from the helical tail. Further attempts to pinpoint single amino acids that determine membrane localization failed to produce conclusive results.

It is not clear at present how the C-terminal helix could affect membrane localization. However, it has been reported that binding of PLCδ$_1$PH to $PI(4,5)P_2$-containing lipid vesicles (but not to soluble $Ins(1,4,5)P_3$) was increased by Ca^{2+} when the C-terminally adjacent EF-hand motifs were added to the PH domain (53). This indicates a conformational change that affects lipid binding and that is initiated via the C-terminal sequences. It has also been reported that a point mutation within the PH domain of PLCδ$_1$ greatly increased the catalytic activity of the enzyme (54), further suggesting a reciprocal communication between the various domains of the PLCδ$_1$ protein. It is also possible that a putative interacting protein contributes to the membrane localization of the PLCδ$_1$PH domain. Further studies will be needed to clarify these questions and to identify a possible protein-binding partner. Several PH domains have been shown to bind βγ subunits of heterotrimeric G-proteins (16, 18) including several PLC isoforms and even PLCδ$_1$PH itself (55, 56). In the few cases where their sites of interaction were mapped, the determinants to bind inositides and those with βγ subunits did not completely overlap, the latter mostly being found toward the C-terminal end of the PH domain (56, 57). Intriguingly, a recent detailed study (58) localized some key residues important for βγ binding of GRK2 to the C-terminal helix of its PH domain. Therefore, βγ subunits are possible candidates to interact with PLCδ$_1$PH to aid its membrane localization. However, overexpression of a construct containing the βγ-binding region of β-adrenergic receptor kinase (59) to sequester βγ-subunits did not significantly affect the membrane localization of PLCδ$_1$PH-GFP.[3]

In a recent study, the p130PH-GFP fusion protein (but not the full-length p130-GFP) was found to localize to the membranes in Madin-Darby canine kidney cells (51). The p130PH domain used in those studies was fused to the C terminus of GFP as opposed to our initial construct in which it was in the N terminus of GFP. This prompted us to create the same GFP-p130PH protein that was used in the studies of Takeuchi *et al.* (51) and to use additional cells types, including Madin-Darby canine kidney cells, to test the distribution of the protein. None of the p130PH domain constructs showed membrane localization in our studies in any of the cells tested, and we have no explanation for the discrepancy between our findings and those of Takeuchi *et al.* (51). However, it is important to note that the lack of membrane localization in our experiments was observed with the same construct that showed $InsP_3$ as well as $PI(4,5)P_2$ binding comparable with those observed with PLCδ$_1$PH-GFP.

Finally, our results also demonstrate that it is possible to use isolated protein domains to manipulate the level of inositol phosphates within intact cells with clearly detectable consequences on Ca^{2+} signaling. Such an approach has been reported recently (60) and will be very useful for the analysis of $InsP_3$-mediated signaling and to give estimates on the free $InsP_3$ levels that are present in intact cells. These data also point to the importance of $InsP_3$ buffering inside the cells and may explain why the estimated $InsP_3$ concentrations (based on total mass measurements) have been found so high (even in resting cells) that they would saturate the $InsP_3$ receptor (61). Therefore, these novel tools to manipulate $InsP_3$ changes could add to our understanding on the physiology of this second messenger.

In summary, using the PH domains of PLCδ1 and the p130 protein, as well as the $InsP_3$ binding domain of the type I $InsP_3$ receptor, we demonstrate that binding to the inositol phosphate head group of $PI(4,5)P_2$ by the PLCδ$_1$ PH domain is necessary but may not be sufficient to localize this protein to cellular membranes. Additional regions within the C-terminal half of the PH domain, namely the loop between the β6 and β7 sheets and the C-terminal α-helix, are needed for this function. Exploration of the structural features that determine PH domain interaction with $InsP_3$, $PI(4,5)P_2$, and with the intact cell membrane could yield valuable information on the regulation of the PLCδ enzymes and help to facilitate the development of strategies for selective inhibition of various inositide-based regulatory processes.

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